REFERENCES

Awapara, J. (1949). *J. biol. Chem.* **178**, 113.
Bull, H. B., Hahn, J. W. & Baptist, V. R. (1949). *J. Amer. chem. Soc.* **71**, 550.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Fowden, L. (1949), cited by Woiwod, A. J. (1949). *Faraday Soc. Disc.* **7**, 331.
Fowden, L. & Penney, J. R. (1950). *Nature, Lond.*, **165**, 846.
Gal, E. M. (1950). *Science*, **111**, 677.
Martin, A. J. P. & Mittelmann, R. (1948). *Biochem. J.* **43**, 353.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Naftalin, L. (1948). *Nature, Lond.*, **161**, 763.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Polson, A. (1948). *Biochem. Biophys. Acta*, **2**, 575.
Ruhemann, S. (1911). *J. chem. Soc.* **99**, 792.
Teeters, W. O. & Shriner, R. L. (1933). *J. Amer. chem. Soc.* **55**, 3026.
Woiwod, A. J. (1949). *Biochem. J.* **45**, 412.

The Enzymic Oxidation of Pyridoxal by Liver Aldehyde Oxidase

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Huff & Perlzweig (1944) have shown that pyridoxic acid (3-hydroxy-5-methoxy-2-methylpyridine-4-carboxylic acid) is normally present in human urine, and that the amount excreted may be increased by the intake of vitamin B_6, usually administered in the form of pyridoxine. As pyridoxal phosphate is known to be active as a coenzyme of amino-acid decarboxylases, it was reasonable to assume that the acid might be produced in the body by a direct oxidation of the aldehyde. In this paper we have been able to show that pyridoxal can, in fact, be oxidized in the presence of liver aldehyde oxidase and that the reaction product is pyridoxic acid. The course of the reaction has been followed by measuring the rate of disappearance of the aldehyde and the accompanying increase in fluorescence, and the reaction product has been isolated and identified as pyridoxic acid.

EXPERIMENTAL

Most of the enzymic studies were carried out using aldehyde oxidase (Knox, 1946) prepared from rabbit liver according to the method of Gordon, Green & Subramanyan (1940). This method was, however, far from satisfactory as the potency and stability of different preparations varied unaccountably. The enzyme seldom remained active for longer than 1 week and frequently lost its activity within a few hours after preparation. Moreover, on numerous occasions, the final $(NH_4)_2SO_4$ precipitate contained a heavy white sediment which could not be redissolved and appeared to adsorb the enzyme irreversibly, so that it was impossible to obtain an active preparation. Although it is not possible to state a definite reason, it appears that in general this occurred when the liver used was unusually heavy and light in colour. On such occasions all the soluble fractions were very turbid, and the final $(NH_4)_2SO_4$ precipitate was dense and almost colourless. Activity measurements at the different stages of the purification process have shown that in these cases the enzyme was originally present in the liver homogenate and was carried down with the $(NH_4)_2SO_4$ precipitate. One suggestion is that the difficulty in separating the enzyme might be due to the presence of large quantities of glycogen.

Because of these difficulties we have, in addition, used a preparation of aldehyde oxidase from horse liver for the isolation of the end product. This enzyme, although less fractionated than the rabbit-liver preparation, had retained its potency during 4 months' storage in the refrigerator, but the high concentration of proteins in the preparation rendered it unsuitable for reaction studies in the fluorimeter.

Crystalline pyridoxal hydrochloride (Merck) was used for the substrate solutions. A stock solution containing 10 mg./ml. remained stable for about 1 month when kept in the refrigerator without exposure to light. Fresh dilutions of this solution were prepared for each test, and all tests were carried out in tubes wrapped in tin foil to minimize the effect of light. The enzymic oxidation of pyridoxal was demonstrated by the use of three independent methods.

Decolorization of methylene blue. This method was used only in preliminary tests to show that pyridoxal undergoes oxidation in the presence of aldehyde oxidase. Determinations were carried out in evacuated Thunberg tubes which contained the substrate in 0·1 M-phosphate buffer, pH 7, in the main tube and the enzyme in the side arm. After mixing, the oxidation was followed by measuring the decolorization of the methylene blue through the decrease in absorption at 660 mμ., using the Coleman Junior spectrophotometer. No decolorization was observed in blank tubes containing no enzyme. In this way pyridoxal was shown to be oxidized in the presence of the two liver preparations mentioned. Xanthine oxidase, however, appeared to be inactive towards pyridoxal.

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Determination of the aldehyde concentration during the course of the oxidation. The aldehyde concentration can be simply determined by addition of alkali to the 2:4-dinitrophenylhydrazone. A pink colour, proportional in depth to the amount present, is then produced (Kalckar, Kjeldgaard & Klenow, 1950). In the case of pyridoxal, the resulting colour is unstable, so that it was necessary to take readings at carefully timed intervals after addition of the alkali. This factor reduces the accuracy of the method, but it was possible to obtain fairly good, comparative results.

To follow the course of an oxidation, samples containing up to 10 μg. of pyridoxal were taken from an enzyme incubation mixture and added to 0·2 ml. of 0·5% 2:4-dinitrophenylhydrazine in 0·1 N-HCl. Water was added to bring the volume up to 1 ml., and the solution was heated to boiling. After cooling, 3 ml. of 2 N-NaOH were added, and the colour was read exactly 30 sec. later in the Coleman spectrophotometer at 500 mμ. A blank sample and a standard containing 10 μg. of pyridoxal hydrochloride were included in each set of determinations.

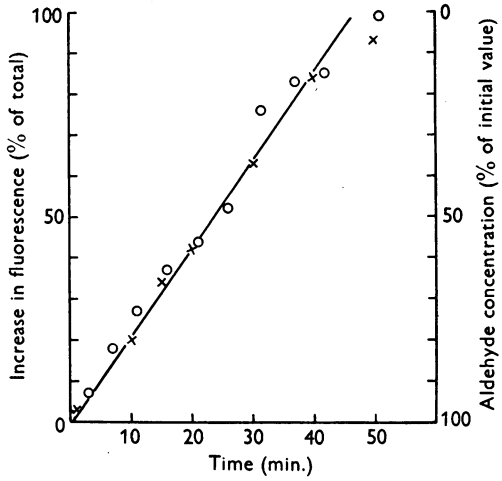

Fig. 1. Relation between the increase in fluorescence and the decrease in aldehyde concentration during oxidation. Pyridoxal hydrochloride (1 mg./ml.) (0·2 ml.) was incubated with 0·8 ml. of rabbit-liver aldehyde oxidase and 3 ml. of 0·1 M-phosphate buffer pH 7. For aldehyde determinations 0·2 ml. samples were pipetted into 0·2 ml. of 0·5% 2:4-dinitrophenylhydrazine. The colour was developed as described in the text. Water was added to both standard and blank so that each tube contained 4 ml. For fluorescence determinations 0·1 ml. samples were pipetted into 1 ml. of phosphate, pH 7, and readings were taken immediately. ○, fluorescence; ×, aldehyde concentration.

As the method was neither sensitive nor accurate enough to be of use in the detailed study of the oxidation, its chief value lay in establishing the validity of using the increase in fluorescence as a measure of the reaction. By measuring both the aldehyde concentration and the fluorescence during the course of a reaction, it was possible to show that the decrease in aldehyde concentration was accompanied by a parallel increase in fluorescence. This relationship is illustrated in Fig. 1.

Fluorescence measurements. Although the fluorescence of pyridoxic acid is almost at its lowest at pH 7, it is still far stronger than that of pyridoxal at the same pH, so that the total increase during a reaction is about tenfold. Measuring this increase is therefore a sensitive and easy method of following the course of an oxidation by continuous observations.

Measurements were carried out in a Farrand multiplier fluorimeter which was set to the required sensitivity with a standard solution of quinine sulphate (0·03 μg./ml.). An appropriate volume of enzyme was added to the substrate contained in 0·1 M-phosphate buffer pH 7, and the fluorescence was recorded at timed intervals. The most convenient substrate concentration was 5–15 μg. of pyridoxal hydrochloride/ml., and, in general, an attempt was made to use an amount of rabbit liver aldehyde oxidase which would complete the reaction within 30 min. when acting on 10 μg. of substrate. Under such conditions the volume of enzyme used was generally 10–50 μl., depending on the potency of the preparation. A blank containing no enzyme never showed any considerable change in fluorescence at pH 7.

RESULTS

By means of fluorimetric measurements the pH optimum of the reaction was established. Since the fluorescence of the resulting acid varies greatly with pH it was necessary to allow the reaction to go to completion, and the rate of reaction during the first 5 min. was expressed as a percentage of the total increase in fluorescence. As in the case of other aldehydes which are oxidized by liver aldehyde oxidase, the optimum pH lies near 7 (Fig. 2).

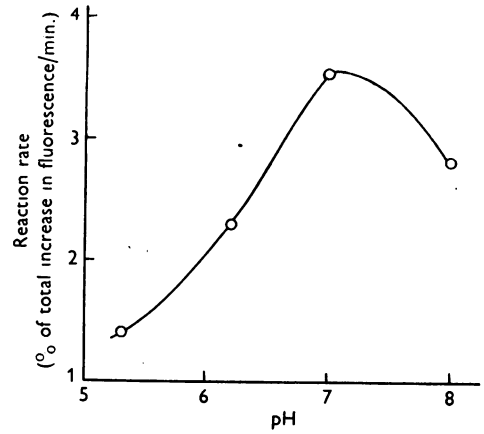

Fig. 2. Rate of oxidation of pyridoxal by liver aldehyde oxidase at different pH values as measured by the rate of increase in fluorescence, which was followed after 0·05 ml. of rabbit-liver aldehyde oxidase had been added to 5 μg. of pyridoxal hydrochloride in 1 ml. of 0·1 M-phosphate buffer of various pH values. The reaction was allowed to go to completion, and the reaction rate is expressed as percentage of the total increase per min. during the initial 5 min. of reaction. In this, as also in other experiments, the standard was arbitrarily set to a fluorescence value of 50 units.

Inhibition by antabuse

It has been shown that antabuse (tetraethylthiuram disulphide) (Hald & Jacobsen, 1948) inhibits the oxidation of acetaldehyde and other aldehydes by liver aldehyde oxidase (Kjeldgaard, 1949). The same effect has now been demonstrated in the oxidation of pyridoxal, and the influence of increasing concentrations of antabuse on the rate of oxidation is illustrated in Fig. 3.

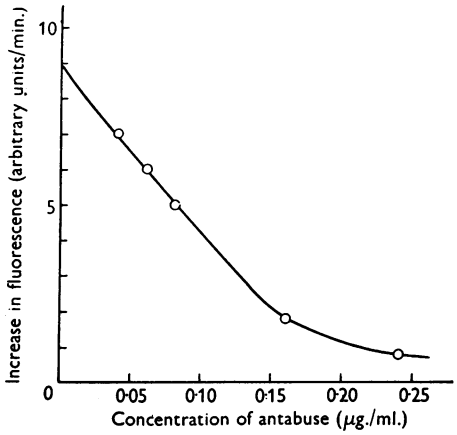

Fig. 3. The effect of varying concentrations of antabuse on the rate of oxidation of pyridoxal. To tubes containing 0·01 mg. of pyridoxal hydrochloride in 1 ml. of 0·1 M-phosphate buffer, pH 7, were added different volumes of an ethanolic solution of antabuse (16 μg./ml.). Each reaction was carried out with 0·02 ml. of rabbit-liver aldehyde oxidase and followed fluorimetrically. The rate of reaction is expressed as the increase in fluorescence per min. during the first 5 min.

The inhibitory effect of antabuse may be reduced by increasing the substrate concentration (Fig. 4). If the rate of oxidation of pyridoxal is plotted against the substrate concentration a curve such as is normal for the majority of enzyme reactions is produced. There is good agreement with the Michaelis-Menten equation over the range recorded, and the Michaelis constant under the conditions of the experiment was found to be $6{\cdot}3 \times 10^{-7}$ M. In the presence of a constant concentration of antabuse the velocity-substrate concentration curve has a similar shape but less slope (curve *B*), so that the Michaelis constant at a concentration of 0·125 μg. of antabuse/ml. had increased to a value of $4{\cdot}1 \times 10^{-6}$ M. When the reciprocal velocity was plotted against reciprocal substrate concentration two straight lines were obtained which met on the *y*-axis (Fig. 5), showing that the velocity at infinite substrate concentration is unaffected by the presence of antabuse. From this it may be concluded that the inhibition by antabuse is of a competitive nature.

Isolation and identification of the end product

Preliminary tests carried out on the product of a reaction whose course to completion had been followed fluorimetrically showed that: (1) The

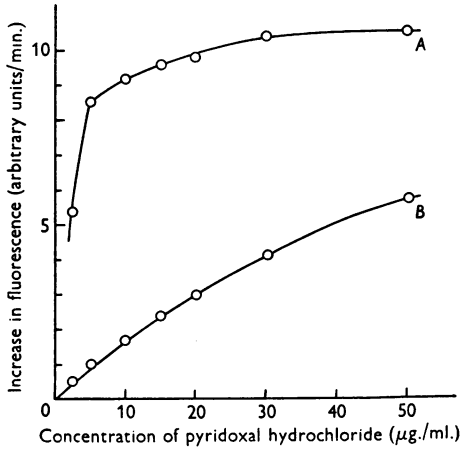

Fig. 4. Effect of substrate concentration on the oxidation of pyridoxal in absence and presence of antabuse. Different volumes of pyridoxal hydrochloride (0·1 mg./ml.) were added to 0·1 M-phosphate buffer, pH 7 + 0·005 ml. of ethanol to give a final volume of 1 ml. The reaction was carried out with 0·015 ml. of rabbit-liver aldehyde oxidase (curve *A*). For curve *B* the reactions were carried out in the same way, but to each tube was added 0·005 ml. of a solution of antabuse in ethanol to give a final concentration of 0·125 μg./ml.

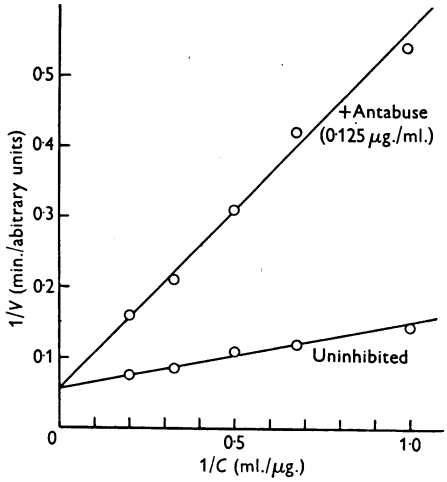

Fig. 5. To demonstrate the competitive nature of the antabuse inhibition. The experimental conditions were the same as for fig. 4: *V*, reaction velocity; *C*, pyridoxal hydrochloride concentration.

product was soluble in butanol and insoluble in ether. (2) The fluorescence varied with pH, with a maximum value at pH 3–4. (3) In 0·5 N-hydro-

chloric acid, after heating in a boiling-water bath for 30 min. the fluorescence greatly increased and the maximum value now occurred at pH 9. As all these properties coincided with those mentioned by Huff & Perlzweig (1944) in the description of pyridoxic acid, we proceeded to isolate the end product by the method described by these authors.

Pyridoxal hydrochloride (100 mg.) was dissolved in 10 ml. of 0·1 M-phosphate buffer pH 7, and incubated with 2 ml. of horse-liver enzyme at 34°. After 24 hr. the reaction was considered to have reached completion as there was no detectable aldehyde present and the fluorescence had reached a steady value. To remove the enzyme the mixture was saturated with Na_2SO_4 and heated to boiling. After filtering and washing the residue with hot water, the clear, yellow solution, which contained about 86 mg. of the acid as indicated by fluorescence measurements, was evaporated to dryness in a current of air. The yellow residue was extracted five times with 10 ml. portions of hot ethanol, and the extract was again evaporated to dryness. The extractions were repeated twice with progressively smaller volumes of ethanol. The final residue was dissolved in 3 ml. of hot 0·1 N-NaOH. After cooling, a few drops of 5 N-HCl were added, when small white crystals began to appear. These were filtered off after standing in the refrigerator overnight, washed rapidly with distilled water, and twice recrystallized from pure pyridine.

The crystals resembled small needles; they were slightly soluble in water, easily soluble in alkali, and insoluble in acid except on prolonged heating when conversion to the far more soluble lactone took place, as indicated by an increased fluorescence. The melting point of the acid was around 245–248° (uncorr., decomp.). The mixed melting point with pyridoxic acid prepared by Huff & Perlzweig was identical. Further comparisons were made, establishing the pH-fluorescence relationships of the two acids and their lactones (Table 1). The ultraviolet absorption spectra of both the acids and their lactones were also found to be identical, although the absolute maximum absorption of the enzymic oxidation product was somewhat lower than that of the synthetic acid. Fig. 6 shows the absorption spectrum of pyridoxal together with those of enzymically formed pyridoxic acid and its lactone, all at pH 7·16.

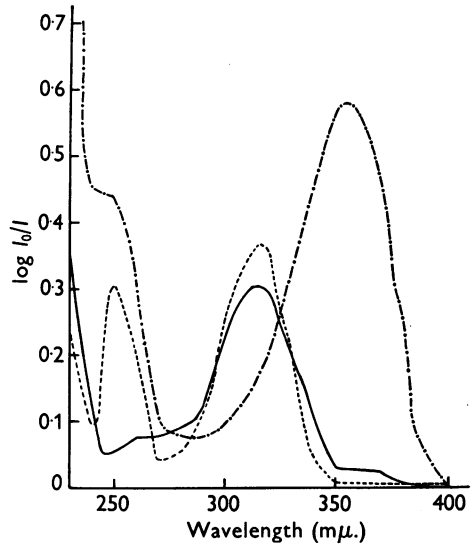

Fig. 6. Absorption spectra of pyridoxal and of enzymically formed pyridoxic acid and its lactone. The solutions contained 10 μg./ml. of pyridoxal hydrochloride, pyridoxic acid and the lactone (prepared as previously described) in 0·2 M-phosphate, pH 7·16. The ultraviolet absorption was measured in the Beckman spectrophotometer. ——, pyridoxic acid; - - - -, pyridoxal hydrochloride; —·—·—, pyridoxic acid lactone.

Table 1. *Comparison of synthetic and enzymically produced pyridoxic acids and their lactones by determination of the fluorescence at various* pH *values*

(Pyridoxic acid (1·11 mg.) and the enzymic reaction product (1·26 mg.) were dissolved in 10 ml. of water. Of this solution 0·01 ml. was added to 1 ml. of buffer for readings in the fluorimeter. For lactone formation 2 ml. of the above acid solution were heated with 0·5 ml. of 2 N-HCl for 30 min., diluted to 10 ml. and 0·01 ml. of the final solution was added to 1 ml. buffer as before.

Buffer solutions: pH 1, 0·1 N-HCl; pH 2–8, McIlwaine standard buffer; pH 9, 0·1 M-sodium pyrophosphate.)

	Acid			Lactone		
pH	Synthetic	Enzymic	Standard setting	Synthetic	Enzymic	Standard setting
1	29	33	25	—	—	—
2	45	50	25	—	—	—
3	44·5	47	25	10	10	50
4	41	45	25	11	11	50
5	23	26	25	18	20	50
6	15	16	25	49·5	54	50
7	11	13	25	74	83	50
8	11	12	25	62 (124)	64 (128)	25
9	—	—	—	74 (370)	88 (440)	10

Figures in brackets are calculated to give the values as if the standard setting had been at 50 throughout.

SUMMARY

1. Pyridoxal can be oxidized by liver aldehyde oxidase.

2. The oxidation is competitively inhibited by antabuse.

3. The end product of the reaction has been isolated and identified as pyridoxic acid.

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REFERENCES

Gordon, A. H., Green, D. E. & Subramanyan, V. (1940). *Biochem. J.* **34**, 764.
Hald, J. & Jacobsen, E. (1948). *Acta Pharmacol.* **4**, 305.
Huff, I. W. & Perlzweig, W. A. (1944). *J. biol. Chem.* **155**, 345.
Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1950). *Biochim. Biophys. Acta*, **5**, 586.
Kjeldgaard, N. O. (1949). *Acta Pharmacol.* **5**, 397.
Knox, W. E. (1946). *J. biol. Chem.* **163**, 699.

The Amino-Acid Composition of Two Yeasts used to Produce Massive Dietetic Liver Necrosis in Rats

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Although yeast consists mainly of protein and is easy and cheap to produce in bulk, it is not a good source of dietary protein. For example, Hock & Fink (1943*a*, *b*) showed that when low-protein diets containing different types of yeasts as the main source of protein were fed to young rats, the animals failed to grow normally and after some time died from acute liver lesions consisting of extensive necrosis and haemorrhages. The yeasts varied both in their growth-promoting properties and in their tendency to produce liver lesions (necrogenic properties); there appeared to be some relation between the sulphur/nitrogen ratio of the yeasts and their necrogenic properties, the more necrogenic diets having lower proportions of sulphur. Liver necrosis in rats has also been produced by feeding diets containing suboptimal amounts of other proteins, such as casein or soya-bean meal, as the main source of protein. The lesions appear to be caused by a condition similar to that occurring in rats fed the yeast diets, since development of necrosis on all these diets could be prevented by extra cystine or methionine (Weichselbaum, 1935; Hock & Fink, 1943*a*; Matet, Matet & Fridenson, 1947; Himsworth & Glynn, 1944*b*; György & Goldblatt, 1949).

Necrosis has been produced in this laboratory by using low-protein diets containing baker's yeast as the sole source of protein (Himsworth & Glynn, 1944*a*). Similar diets containing brewer's yeast have been shown by György to be considerably less necrogenic (György, 1948; György & Goldblatt, 1949). Since it seemed probable that the difference in types of yeast used in the diets accounted for the different results obtained in the two laboratories, samples of yeast were exchanged in order to compare their chemical composition and their nutritional properties for the same strain of rat.

The chemical investigation was confined to the amino-acid fractions, particularly sulphur-containing amino-acids. Tocopherol deficiency is another factor involved in the production of necrosis (Schwarz, 1944; Himsworth & Lindan, 1949), but since tocopherol is known to be absent from all types of yeasts (György, 1948, private communication) it could not have accounted for the differences observed. This paper describes the results of investigations on the nitrogen, sulphur and amino-acid contents of our baker's yeast and György's brewer's yeast. Because of the possibility that the extractable and therefore immediately available amino-acids and peptides might be of importance in the nutritional properties of the yeasts, separate investigations were made on the amino-acids present in the cell residues insoluble in 75% ethanol and on